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The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review

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ABSTRACT

A growing number of publications supports a biologic effect of the protein-bound uremic retention solutes indoxyl sulfate and p-cresyl sulfate. However, the use of unrealistically high free concentrations of these compounds and/or inappropriately low albumin concentrations may blur the interpretation of these results. Here, we performed a systematic review, selecting only studies in which, depending on the albumin concentration, real or extrapolated free concentrations of indoxyl sulfate and p-cresyl sulfate remained in the uremic range. The 27 studies retrieved comprised *in vitro* and animal studies. A quality score was developed, giving 1 point for each of the following criteria: six or more experiments, confirmation by more than one experimental approach, neutralization of the biologic effect by counteractive reagents or antibodies, use of a real-life model, and use of dose–response analyses *in vitro* and/or animal studies. The overall average score was 3 of 5 points, with five studies scoring 5 of 5 points and six studies scoring 4 of 5 points, highlighting the superior quality of a substantial number of the retrieved studies. In the 11 highest scoring studies, most functional deteriorations were related to uremic cardiovascular disease and kidney damage. We conclude that our systematic approach allowed the retrieval of methodologically correct studies unbiased by erroneous conditions related to albumin binding. Our data seem to confirm the toxicity of indoxyl sulfate and p-cresyl sulfate and support their roles in vascular and renal disease progression.

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As CKD evolves, uremic retention becomes a progressively more important contributor to overall organ dysfunction.[1–3] Among the three physicochemical types of uremic solutes,[4] the pathophysiologic importance of protein-bound toxins has been neglected for a long time,[5–7] leading to a shortage of specific removal strategies.[8,9] In the past decade, however, a growing number of publications documented the impact of protein-bound uremic toxins on vital processes and an association of their concentration with clinical outcome parameters.[6,10–19]

One factor blurring the interpretation of the biochemical effects of uremic toxins is the application of unrealistic concentrations compared with the concentrations observed in human CKD in *in vitro* testing or *in vivo* animal experiments.[4,20] Moreover, for protein-bound toxins, even with seemingly acceptable total concentrations, the quantities of albumin or protein present are often too low, resulting in unacceptably high and thus, irrelevant free concentrations.

In analogy to drugs, albumin is likely to act as a buffer, attenuating potential biochemical effects of its ligands,[21–23] whereas recent data clearly showed that, for both indoxyl sulfate and p-cresyl sulfate, albumin is the main binding protein.[24]

Absence of appropriate albumin quantities results in too high free solute concentrations, inducing exaggerated biologic responses with a potential for an overestimated toxic impact. This bias has provoked justified objections against recognizing protein-bound molecules as real uremic toxins. In a recent editorial,[25] indoxyl sulfate, one of the main protein-bound solutes, was for that reason called "long suspected but not yet guilty."

Nevertheless, a careful check of the literature reveals that investigators in a number of studies have applied acceptable free concentrations by administering those toxins to animals up to acceptable total concentrations, adding relevant quantities of albumin to *in vitro* media by using whole blood, plasma, or serum, or in the absence of albumin, applying concentrations corresponding to the free uremic fraction. At this moment, it is unclear, however, how many methodologically suitable publications are available and what they teach us about the contribution of the compounds to the uremic syndrome.

In the present analysis, we applied a systematic search to filter out those studies in which unacceptably high free concentration of protein-bound solutes had been applied. We aimed to analyze the effect of indoxyl sulfate and p-cresyl sulfate *in vitro* and in animals. Based on

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preset standards, 27 articles of adequate quality were retrieved and are reported here together with the shown effects.

RESULTS

The literature was searched according to preset methods (see below) to retrieve studies on biologically relevant toxic effects of indoxyl sulfate and p-cresyl sulfate following well defined quality standards. One of the conditions was that the concentrations used in those studies conformed with the concentrations observed in uremia, which is illustrated in Table 1. The concentrations considered appropriate were in the high uremic range to compensate for the often short exposure time in the experimental studies, especially *in vitro*. To make a comparison with the values currently observed in an average dialysis population, we give in Table 1, also as reference, data retrieved from a recent study from the European Uremic Toxin Work Group as a real-life orientation for the reader.[20] We identified 336 citations through electronic searching and added 61 citations from reference lists of retrieved publications (Figure 1). Based on the selection criteria, we included 27 studies in the review (Table 2).[26–52]

The number of retrievable studies increased considerably over the past years, suggesting a rising awareness about the topic. The distribution among countries points to a broad worldwide interest. The majority (n=20) was generated in Asia, especially Japan; however, seven studies in total were performed in Europe, the United States, or Australia.

The following cell and organ systems were investigated (n of each study is in parentheses) (Table 2): renal tubules (9),[34,36–39,43,45,47,50] endothelium (6),[26–30,33] leukocytes (2),[30,31] whole vessels (2),[40,41] whole kidney (2),[40,42] cardiac cells (2),[35,49] smooth muscle cells (2),[46,51] hepatocytes (2),[32,51] intestinal cells (1),[48] and adipocytes (1).[44] A few studies concentrated on more than one target system: one study was on both the whole vessel and kidney[40] and one study was on leukocyte–endothelial interaction.[30]

Twenty-four studies concentrated on indoxyl sulfate,[26–30,32–43,46–52] and six studies concentrated on p-cresyl sulfate.[31,34,36,39,44,45] Three studies evaluated both compounds.[34,36,39] Some studies also assessed alterations induced by other uremic toxins, but only two of those studies showed a significant effect for indole acetic acid,[46,48] hippuric acid,[48] and carboxy-methyl-propyl-furanpropionic acid.[48]

All studies evaluating indoxyl sulfate showed a negative (toxic) effect (Table 3), except the study by Odamaki *et al.*,[52] which showed an increase of hepatocyte albumin production in the presence of indoxyl sulfate. Four studies on p-cresyl sulfate[53–56] and one study on indoxyl sulfate[55] showed no significant changes (data not shown).

Of 14 animal studies, 10 studies were in rats,[37,38,40–43,45,47,49,50] and four studies were in mice.[30,36,39,44] Several of the rat studies were in the specific Dahl salt-sensitive hypertensive rat strain,[37,38,40–43,47,49,50] although in some of these studies, an effect in the wild type was also observed.[37,38,43,47,50] One study only evaluated Dahl wild-type rats and found a significant effect of indoxyl sulfate in that strain.[42]

Of the *in vitro* studies, eight studies applied an appropriate concentration of albumin, resulting in relevant free toxin concentrations,[26–29,31,46,51,52] whereas in the remaining seven studies, a concentration compatible with the uremic free fraction was pursued.[32–36,39,48]

A large array of mediators, pathways, and messenger molecules contributed to the observed system effects (Table 4). Several were confirmed in more than one study of this series.[27,30,34–36,38,40,43,45,48–50] Several of the observed effects were related to inflammation/free radical production[27,30,34–36,44,45,48–50] and fibrosis.[36,38–40,43,49] The mutual connections of these factors into global pathways for endothelial and proximal tubular cells are shown in Figure 2.

With regards to quality, all retained studies together had a median score of 3.0 from a possible total of 5 points, indicating that approximately one half of the retained studies had a score of 3 points or above (Supplemental Material 1). Five studies reached a score of 5 of 5 points,[35,36,39,44,45] and six studies obtained a score of 4 of 5 points.[27,30,38,46,49,50] These 11 studies with a high quality score covered a broad array of pathophysiologic mechanisms mainly related to cardiovascular damage and progression of kidney disease: reactive oxygen species generation (3),[27,45,50] endothelial dysfunction (2),[27,30] epithelial-to-mesenchymal transition (2),[36,38] leukocyte–endothelial interaction (1),[30] deterioration of cardiac cell functional capacity (1),[35] Klotho expression (1),[39] expression of tissue factor in vascular smooth muscle cells (1),[46] and insulin resistance (1).[44] The reasons for scoring 4 of 5 points instead of 5 of 5 points were lack of proof of concept by effect neutralization two times, the number of experiments was too low two times, lack of confirmation by an alternative approach one time, and absence of real-life conditions one time. There was no significant

Table 1. Threshold values of concentration

	Normal (mg/L)	Uremia (mg/L)	Comments
IS (molecular weight 212)			
IS_{total} maximum	—	236.0[4]	Highest individual value
IS_{total} high	0.5[20]	44.5[20]	Highest mean
IS_{free} high	Less than LOD	4.5[20]	Highest mean
PCS (molecular weight 188)			
PCS_{total} maximum	—	105.0[78]	Highest individual value
PCS_{total} high	2.9[77]	43.0[78]	Highest mean
PCS_{free} high	0.1[20]	2.6[20]	Highest mean

Highest individual value is the highest single value per patient ever reported; highest mean is the highest mean for a patient group ever reported. Current average values observed in an average dialysis population as reported in the most recent review of the European Uremic Toxin Group are 23.1 mg/L for IS_{total} and 20.9 mg/L for PCS_{total}.[20] IS, indoxyl sulfate; LOD, limit of detection; PCS, p-cresyl sulfate.

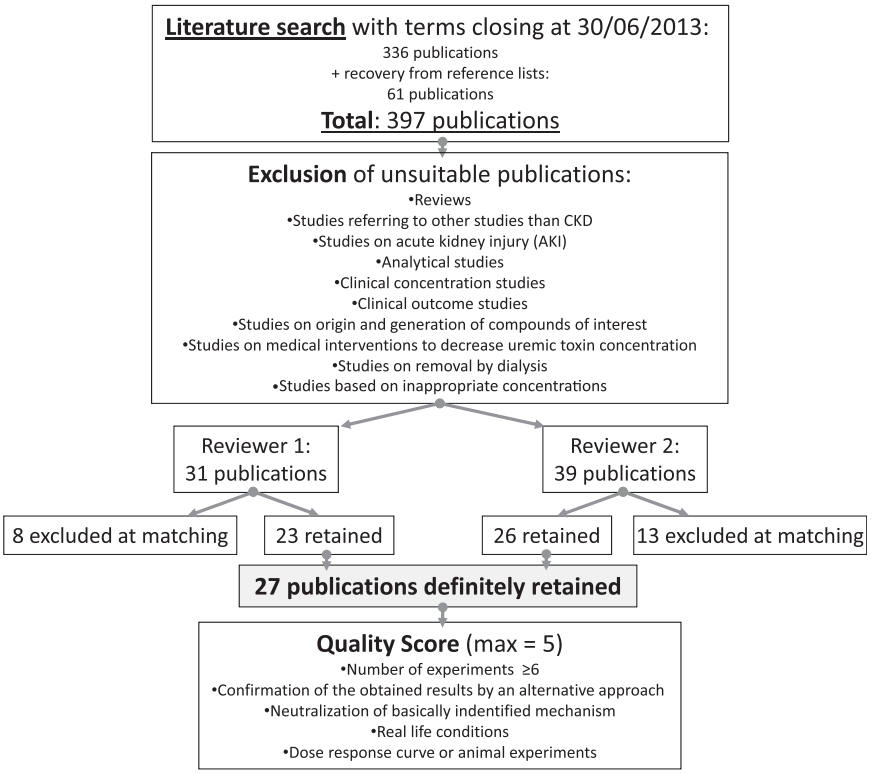

Figure 1. Flowchart of the review procedure and quality scoring.

correlation between year of publication and quality score (data not shown).

DISCUSSION

This study reviews the literature evidence up until June 30, 2013, for the biologic and/or toxic effects of two prototype protein-bound uremic toxins: indoxyl sulfate and p-cresyl sulfate. In total, 27 studies from all over the world were retained, with the principal topics being endothelial and proximal tubular cell dysfunction, although insulin resistance, functional status of cardiac cells, leukocyte dysfunction, coagulation disturbances, and pharmacodynamics were also covered. A quality assessment identified 11 of 27 (41%) studies with a score of at least 4 points from a possible maximum of 5 points. The majority of the changes found affects mechanisms that are essential to the pathophysiology of the uremic syndrome and its subsequent deleterious impact on prognosis.

Endothelial dysfunction,[26–30,33] smooth muscle cell lesions,[46,51] coagulation disturbances,[46] leukocyte activation,[30,31] cross-talk of leukocytes and endothelium,[30] cardiac fibrosis and hypertrophy,[35,49] and insulin resistance[44] as well as whole-vessel alterations *per se*[40,41] are all linked to cardiovascular damage and contribute to the increased propensity for mortality and cardiovascular events in CKD.[57,58] Both indoxyl sulfate and p-cresyl sulfate *per se* have repeatedly been related to cardiovascular mortality.[10–19] Although strategies that more efficiently remove protein-bound solutes, such as frequent dialysis[59] and online hemodiafiltration,[60,61] in controlled studies are associated with improved outcomes,[62,63] it remains difficult to interpret these data because of the presence of confounding factors that affect the outcomes on their own and a lack of selectivity of removal, because dialysis strategies are usually not restricted to protein-bound solutes alone.

Next to the above cardiovascular problems, the data linking these solutes to tubular cell damage,[39,45] tubular epithelial-to-mesenchymal transition,[36,38] tubulointerstitial inflammation and fibrosis,[34,36,37,39,47,50] or whole-kidney damage[40,42] are all are linked to the progression of CKD, another factor related to uremic morbidity and mortality.[57] As a consequence, decrease in concentration of intestinally generated uremic retention solutes, like indole, by the intestinal sorbent AST-120 (Kremezin) has been accepted in mostly Asian countries as a strategy to restrain the progression of CKD. It is very likely that this sorbent affects the concentration of not only indoxyl sulfate but also, other protein-bound uremic toxins, one of which is p-cresyl sulfate.[64] In at least two small-sized randomized controlled trials, AST-120 had a positive effect on progression of CKD.[65,66] In a recent large randomized controlled trial in the United States and Europe, however, this positive effect could not be confirmed.[67]

Until now, biologic data were perceived as not consistent or convincing enough to recognize protein-bound toxins as the real culprits.[25,68,69] The results of the current analysis applying systematic search methods linked to a quality assessment of the retrieved publications, however, yielded several high-quality papers applying, for the uremic setting, acceptable concentrations and state-of-the-art test methods. Therefore, our analysis offers, in our opinion, arguments that are solid enough to reconsider the reluctance to recognize the protein-bound solutes indoxyl sulfate and p-cresyl sulfate as toxic.

This study is, to the best of our knowledge, the first to apply to biologic studies a stringent strategy that had been defined in advance, *in casu* related to uremic retention, resulting in a systematized summary of their toxicity. Although we tried to follow current standards for the conduct of systematic reviews,[70] there are methodological deviations. In contrast to evidence-based systematic reviews, in our case, only a limited number of search engines was used. Of note, for biologic studies, especially in the area of uremia, there is, to the best of our knowledge, not a large repository of data such as the one made available by the Cochrane Library (Cochrane Central Register of Controlled Trials) for clinical studies. Our approach may serve

Table 2. Retained articles and their main characteristics

First Author, Year, and Reference	Cell/Organ System	Toxin	Concentration (mg/L)/Dose	Albumin/Type Model
(*1*) *In vitro*, albumin: normal				
Dou, 2004[26]	Endothelium	IS	25–250	40 g/L
Odamaki, 2004[52]	Hepatocytes	IS	50–100	40 g/L
Faure, 2006[28]	Endothelium	IS	256	40 g/L
Yamamoto, 2006[51]	Smooth muscle cells	IS	53–106	40 g/L
Dou, 2007[27]	Endothelium	IS	125–250	40 g/L
Schepers, 2007[31]	Leukocytes	PCS	121.0	Whole blood
Itoh, 2012[29]	Endothelium	IS	29.9–57.2	40 g/L
Chitalia, 2013[46]	Smooth muscle cells	IS	25	40 g/L
Koppe, 2013[44]	Adipocytes, myotubes	PCS	40	35 g/L
(*2*) *In vitro*, albumin: low, absent, or unspecified				
Tsujimoto, 2010[32]	Liver microsomes	IS	6.36	Absent
Lekawanvijit, 2010[35]	Cardiac fibroblasts/myocytes	IS	0.02–63.6	5 g/L BSA/absent
Yu, 2011[33]	Endothelium	IS	1.25–125	Unspecified
Sun, 2013[34]	Proximal tubular cells	IS	1; 5	Absent
		PCS	1; 5	
Sun, 2012[36]	Proximal tubular cells	IS	1–50	Absent
		PCS	1–50	
Sun, 2012[39]	Proximal tubular cells	IS	1; 5	Unspecified
		PCS	1; 5	
Tsujimoto, 2012[48]	Intestinal cells (hepatic no effect)	IS	4.24	0.8 g/L (10% FBS)
(*3*) Animal[a]				
Adijang, 2008[40]	Aorta, kidney	IS	23.1/200 mg/kg (30 wk) p.o.	DH rat
Ito, 2010[30b]	Endothelium/leukocyte interaction	IS	15.7/200 mg/kg q.d. (10 d) p.o.	Nx mouse
Adijang, 2010[41]	Aorta	IS	15–20/200 mg/kg (32 wk) p.o.	DN, DH rat
Bolati, 2011[38b,c]	Tubular cells	IS	9.4–18.9/200 mg/kg (32 wk) p.o.	DH rat
Sun, 2012[36]	Tubular cells	IS	Unspecified/100 mg/kg q.d. (4 wk) i.p.	1/2 Nx mouse
		PCS		
Watanabe, 2013[45b]	Renal tubular cells	PCS	32.6/50 mg/kg q.d. (4 wk) i.p.	5/6 Nx rat
Sun, 2012[39]	Proximal tubular cells	IS	8.5	1/2 Nx mouse
		PCS	1.8	
			100 mg/kg q.d. (4 wk) i.p.	
Shimizu, 2012[37b,c]	Proximal tubular cells	IS	9.4–18.9/200 mg/kg (32 wk) p.o.	DN, DH rat
Shimizu, 2013[42b]	Kidney (cortex)	IS	9.4/200 mg/kg q.d. (32 wk) p.o.	DN rat
Shimizu, 2013[43b]	Tubular cells	IS	9.4/200 mg/kg (32 wk) p.o.	DN rat
Koppe, 2013[44]	Adipocytes, myocytes, various organs	PCS	51/10 mg/kg b.i.d. (4 wk) i.p.	Normal RF mouse
Shimizu, 2013[47b,c]	Tubular cells	IS	9.4–18.9/200 mg/kg (32 wk) p.o.	DN, DH rat
Yisireyili, 2013[49]	Cardiac tissue	IS	18.9/200 mg/kg q.d. (32 wk) p.o.	DN, DH rat
Bolati, 2013[50b]	Tubular cells	IS	9.4/200 mg/kg q.d. (32 wk) p.o.	DN, DH, rat

IS, indoxyl sulfate; PCS, p-cresyl sulfate; BSA, bovine serum albumin; FBS, fetal bovine serum; wk, weeks; p.o., *per os*; q.d., once a day; i.p., intraperitoneal; b.i.d., twice per day; DH, Dahl salt-sensitive hypertensive; Nx, nephrectomized; DN, Dahl salt-resistance normotensive; RF, renal function.

[a]For animal experiments, we presumed that serum albumin was present at the same concentrations as usually in these animals; all solute concentrations were measured in serum, except for the study by Ito *et al.*[30] (plasma), and steady state at the end of exposure on euthanasia of the animals, except for the study by Koppe *et al.*[44] (peak after i.p. injection). Also, dose and administration route are mentioned; the Albumin/Type Model column contains both species and type of model.

[b]*In vitro* part not appropriate.

[c]If concentration is 9.4–18.9, it is 9.4 for Dahl wild-type rats and 18.9 for DH rats.

as a basis for future analyses of the same kind.

If we consider a quality score of 4 or 5 points of 5 points as the equivalent of a high evidence label, the results reported in the present article should provide a positive incentive to developing strategies to remove these protein-bound molecules better and more consistently than at present. Alternatives to the standard approaches that we now know to have a potentially beneficial impact are extended dialysis,[71] prebiotics,[72,73] probiotics[72,73] and extracorporeal sorbents.[74] Because our knowledge regarding this issue is extending, alternative options may arise in the nearby future. Anyway, the fact that dietary components and residual renal function seem to affect protein-bound solute concentration more than dialysis adequacy, as assessed by Kt/V,[75] may suggest that alternative methods focusing on preserving kidney function or affecting intestinal toxin generation

Table 3. Main relevant pathophysiologic changes per study

Cell/Organ System	Toxin	Main Observed Effect
(*1*) Endothelium		
Dou, 2004[26]	IS	Inhibition proliferation and repair
Faure, 2006[28]	IS	Increase endothelial microparticle release
Dou, 2007[27]	IS	Induction ROS, inhibition glutathione
Ito, 2010[30]	IS	Increase endothelial/leukocyte interaction (adhesion)
Yu, 2011[33]	IS	Inhibition proliferation; increase senescence; increase ROS and decrease NO production
Itoh, 2012[29]	IS	Induction ROS
(*2*) Smooth muscle cells		
Yamamoto, 2006[51]	IS	Increase proliferation
Chitalia, 2013[46]	IS	Generation tissue factor (linked to clot formation), tissue factor breakdown retarded
(*3*) Leukocytes		
Schepers, 2007[31]	PCS	Activation oxidative burst
Ito, 2010[30]	IS	Increase endothelial/leukocyte interaction (adhesion)
(*4*) Whole vessels		
Adijang, 2008[40]	IS	Increase aortic calcification and stiffness, expression osteoblast markers, and OATs
Adijang, 2010[41]	IS	Induction cell senescence
(*5*) Cardiac cells		
Lekawanvijit, 2010[35]	IS	Cardiomyocyte proliferation, cardiac fibrosis, inflammation
(*6*) Whole heart		
Yisireyili, 2013[49]	IS	Increase cardiomyocyte hypertrophy, cardiac fibrosis, oxidative stress
(*7*) Renal tubular cells		
Bolati, 2011[38]	IS	Increase epithelial-to-mesenchymal transition
Sun, 2013[34]	IS, PCS	Enhanced expression cytokine and inflammatory genes
Sun, 2012[36]	IS, PCS	Activation RAAS and epithelial-to-mesenchymal transition, fibrosis, nephrosclerosis
Shimizu, 2012[37]	IS	Increase expression MCP-1
Sun, 2012[39]	IS, PCS	Increase Klotho gene methylation, renal fibrosis
Watanabe, 2012[45]	PCS	Increase renal tubular damage
Shimizu, 2013[43]	IS	Induction mediators for fibrosis (TGF-β_1 and Smad3)
Shimizu, 2013[47]	IS	Induction generation adhesion molecule (ICAM-1)
Bolati, 2013[50]	IS	Increased oxidative stress
(*8*) Whole kidneys		
Adijang, 2008[40]	IS	Increase fibrosis
Shimizu, 2013[42]	IS	Increase angiotensinogen expression
(*9*) Hepatocytes		
Odamaki, 2004[52]	IS	Increase albumin production
Tsujimoto, 2010[32]	IS	Inhibition metabolic clearance losartan
(*10*) Intestinal cells		
Tsujimoto, 2012[48]	IS	Inhibition pumps involved in clearance of pravastatin
(*11*) Adipocytes		
Koppe, 2013[44]	PCS	Increase insulin resistance, decrease lipogenesis, increase lipolysis, ectopic lipid redistribution

IS, indoxyl sulfate; ROS, reactive oxygen species; NO, nitric oxide; PCS, p-cresyl sulfate; OATs, organic anion transporters; RAAS, renin angiotensin aldosterone system; MCP-1, monocyte endothelial chemotactic protein-1; TGF-β_1, transforming growth factor-β_1; ICAM-1, intercellular adhesion molecule-1.

may be as important as extracorporeal removal.

The question could be raised as to how far toxic mechanisms and pathways overlap for both toxins. It is only possible if studies on the two compounds assess the same elements. To the best of our knowledge, for the time, such a parallel approach has only been used in renal tubular cells. Figure 2B confirms that, indeed, some mechanisms are overlapping.

This study has a few drawbacks. First, we focused only on two of the protein-bound solutes, whereas several other compounds in this group might have a biologic impact as well.[3,5,6,76] We selected, however, the two molecules that have been most extensively evaluated. Some of the retained studies also show similar effects for other protein-bound solutes,[46,48] suggesting that the yielded evidence could have been even more convincing if more compounds would have been taken into account. Second, we accepted concentrations up to the upper uremic range (Table 1),[4,20,77,78] whereas in the average uremic patient, the concentration will be lower. However, a high concentration was applied essentially *in vitro*, where the concentration should be considered to compensate for short exposure to uremic toxins, in contrast to real life, where it is continuous, allowing solutes more time to reach the intracellular compartments where biologic activity is exerted. It is also of note that, in the *in vivo* animal studies, depending on the way of administration (intraperitoneally versus *per os*), the

Table 4. Most important affected pathophysiologic mechanisms

Affected Mediators	Effect	Toxin	Confirmed in Two or More Studies	Related to Inflammation	Related to Fibrosis
α-Smooth muscle actin[36,38,40,49]	↑	IS, PCS	X		X
Cytokine generation[34,35]	↑	IS, PCS	X	X	
E-cadherin[36,38]	↓	IS, PCS	X		X
Nuclear Factor (erythroid-derived 2) -like 2[49,50]	↓	IS	X	X	
Extracellular-regulated kinase 1/2[44]	↑	PCS		X	
Fibronectin[36]	↑	IS, PCS			X
Heme oxygenase-1[49,50]	↓	IS	X	X	
Intercellular Adhesion Molecule-1[47]	↑	IS		X	
Inflammatory genes[34]	↑	IS, PCS		X	
Insulin receptor substrate-1[44]	↓	PCS		X	
Klotho[39]	↓	IS, PCS			X
Mitogen-activated protein kinase: MEK 1/2; p38[35]	↑	IS		X	
Multidrug resistance-associated protein-2[48]	↓	IS			
NADPH oxidase[27,30,45]	↑	IS, PCS	X	X	
Nuclear Factor-κB[35]	↑	IS		X	
Organic Anion Transporters[40,48]	↓	IS	X		
Osteoblast-specific proteins[40]	↑	IS		X	
Phosphoinositide 3-kinase[44]	↓	PCS		X	
Protein kinase B/Akt[44]	↓	PCS		X	
Renin-angiotensin-aldosterone system[36]	↑	IS, PCS	X	X	
E-selectin[30]	↑	IS		X	
Senescence proteins[41a]	↑	IS			
Smad2/3 and Smad4[36,43]	↑	IS, PCS	X		X
Snail[36]	↑	IS, PCS			X
Tissue factor[46]	↑	IS			
Transforming Growth Factor-β[36,40,43,49]	↑	IS, PCS	X		X
Zonula occludens-1[38]	↓	IS			X

IS, indoxyl sulfate; PCS, p-cresyl sulfate.
[a]Senescence proteins: senescence-associated β-galactosidase, 16INK4a, p21WAF1/CIP1, p53, and retinoblastoma protein.

dose, and the type of model with regard to kidney function, fluctuating concentrations and different peak values might occur in the serum, which might, in turn, impact the interpretation of the observed biologic effect.

Third, our review data pointed overwhelmingly to a toxic effect of indoxyl sulfate and p-cresyl sulfate that may be skewed by submission bias. Investigators may be tempted not to finalize or publish studies showing no ill effect. Nevertheless, we retrieved five such studies by our analysis.[52–56] Fourth, we should consider the possibility that we missed some studies in our analysis because of the approach followed, but more data would only increase the evidence delivered here.

However, our approach, based on rigid threshold levels, must also have excluded a number of interesting studies based on concentrations just above threshold[77] and/or pathophysiologic key aspects highly relevant to the uremic syndrome, such as endothelial microparticle release,[56] tissue factor activation,[79] or metabolic capacity for glucuronidation.[80]

Because studies on toxicity in tubular cells suggest a relation to progression of kidney failure that is especially important in earlier stages of CKD, it can be argued that lower concentrations should be pursued in these experiments. However, even in dialysis patients, restraining progression may have a substantial clinical impact, because residual renal function is definitely inversely related to mortality.[81] In addition, in most of studies on tubular toxicity that were retrieved,[34,36–39,42,43,45,47,50] concentrations were in the same range as those concentrations found in patients with CKD stages 3–5 not on dialysis[13,82] (*i.e.*, lower than in dialysis patients).

In conclusion, a systematic retrieval approach searching for experimental studies evaluating the biologic (toxic) impact of the uremic solutes indoxyl sulfate and p-cresyl sulfate and taking into account precise criteria for concentrations that considered protein binding yielded 27 publications that conformed with the inclusion criteria.

Eleven of these studies obtained a high-quality score, indicating a substantial scientific impact. Most frequently, missing quality factors were insufficient number of experiments, lack of a dose–response analysis, and especially, absence of data showing the neutralization of the effect found by counteragents of the presumed mechanism. Future studies should comply with these conditions to be considered of high quality. The retrieved studies covered a broad array of effects, most of them related to clinically highly relevant aspects, such as cardiovascular disease or progression of CKD. These data, if matched to observational outcome studies,[6,10–19] are the best evidence that we can obtain right now for the clinical impact of protein-bound

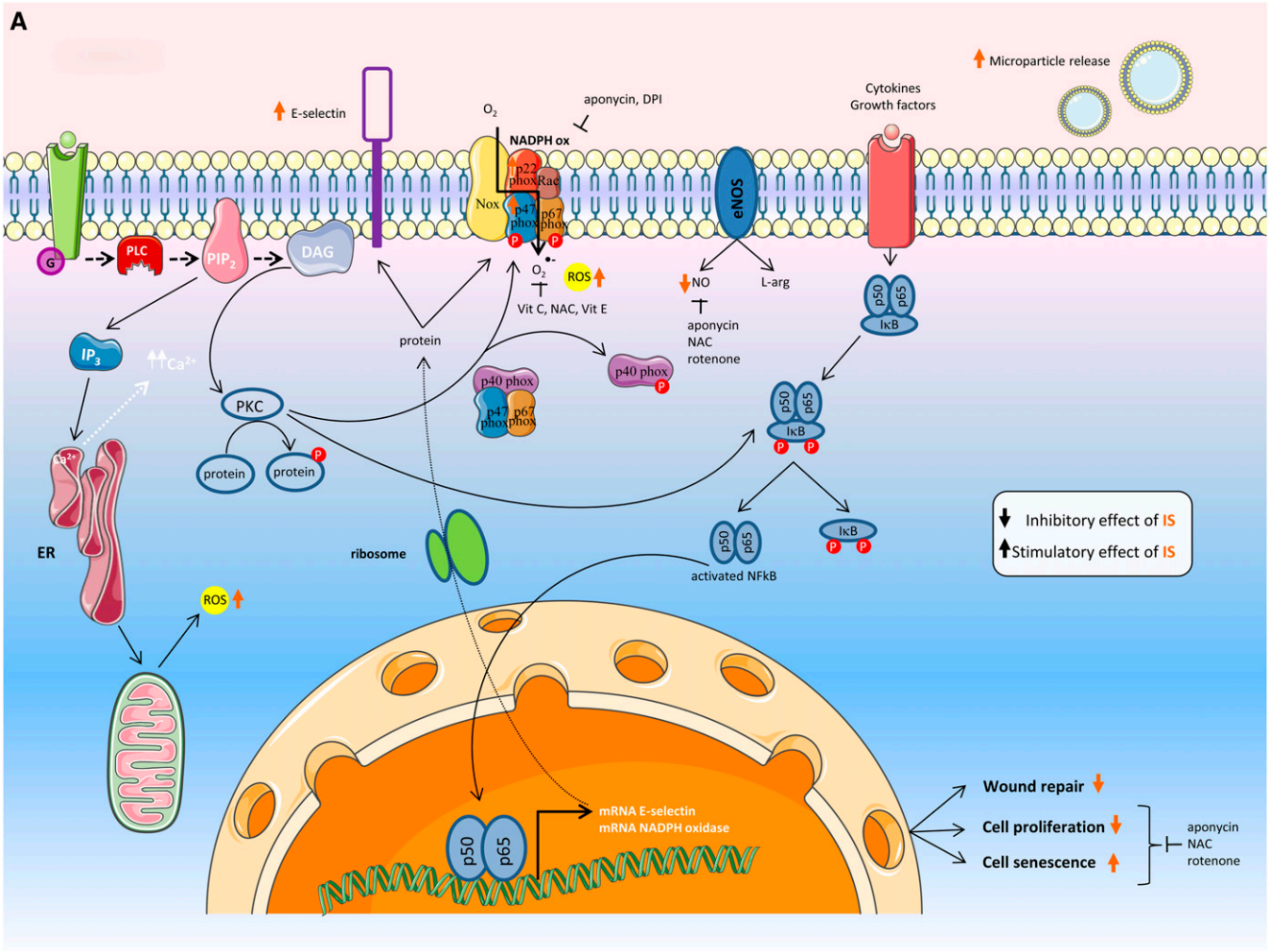

Figure 2. Effects as seen in the (A) endothelial cell and (B) renal tubular cell. The effects of indoxyl sulfate and p-cresyl sulfate are represented by orange and purple arrows, respectively. The transcellular membrane transport of indoxyl sulfate and p-cresyl sulfate by the organic anion transporters is represented by the thick white arrow. α-SMA, α-smooth muscle actin; 5-Aza-2dc, 5-Aza-2′-deoxycytidine; CpG, cytosine-phosphate-guanine (DNA sequence); DAG, diacylglycerol; DNMT, DNA methyltransferase; DPI, diphenylene iodonum chloride; EMT, epithelial-to-mesenchymal transition; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; G, G protein-coupled receptor; HO-1, heme oxygenase-1; ICAM-1, intercellular adhesion molecule-1; IKB, inhibitor of κB; IP_3, inositol 1,4,5-triphosphate; IS, indoxyl sulfate; L-arg, L-arginine; LFA-1, lymphocyte function-associated antigen 1; MCP-1, monocyte chemoattractant protein-1; Me, Methylgroup; NAC, N-acetylcysteine; NADPH ox, NADPH oxidase; NO, nitric oxide; NQO1, NADPH guinone oxidoreductase 1; Nrf, NF (erythroid-derived 2) -like; OAT, organic anion transporter; 8-OHdG, 8-hydroxydeoxyguanosine; p, protein; PCS, p-cresyl sulfate; PIP_2, phosphatidylinositol 4,5-biphosphate; PLC, phospholipase C; PKC, protein kinase C; RAAS, renin angiotensin aldosterone system; ROS, reactive oxygen species; Vit C, vitamin C; Vit E, vitamin E; ZO-1, Zonula occludens protein 1.

solutes. This knowledge may be supportive of the development of strategies to lower the concentration of these solutes in the body.

CONCISE METHODS

The study was limited to indoxyl sulfate and p-cresyl sulfate, the two protein-bound uremic toxins that have been most extensively evaluated until now. A literature search using Pubmed through Reference Manager was undertaken with the following terms: p-cresyl sulfate or p-cresylsulfate, or indoxylsulfate or indoxyl sulfate. This search was completed with additional references emanating from the originally retrieved publications. The search contained all obtained references with June 30, 2013, as closing date (Figure 1).

We only included primary studies assessing the biologic effect of indoxyl sulfate and/or p-cresyl sulfate *in vitro* or in animals where an acceptable free concentration was present. For this reason, analytical studies, clinical concentration or outcome studies, and studies on the origin and generation of the compounds of interest, medical interventions to decrease their concentration, and removal by dialysis or other strategies were all excluded. Also, studies not specifying the pursued solute concentrations or unclear on the applied methods, making reproduction of the experiments

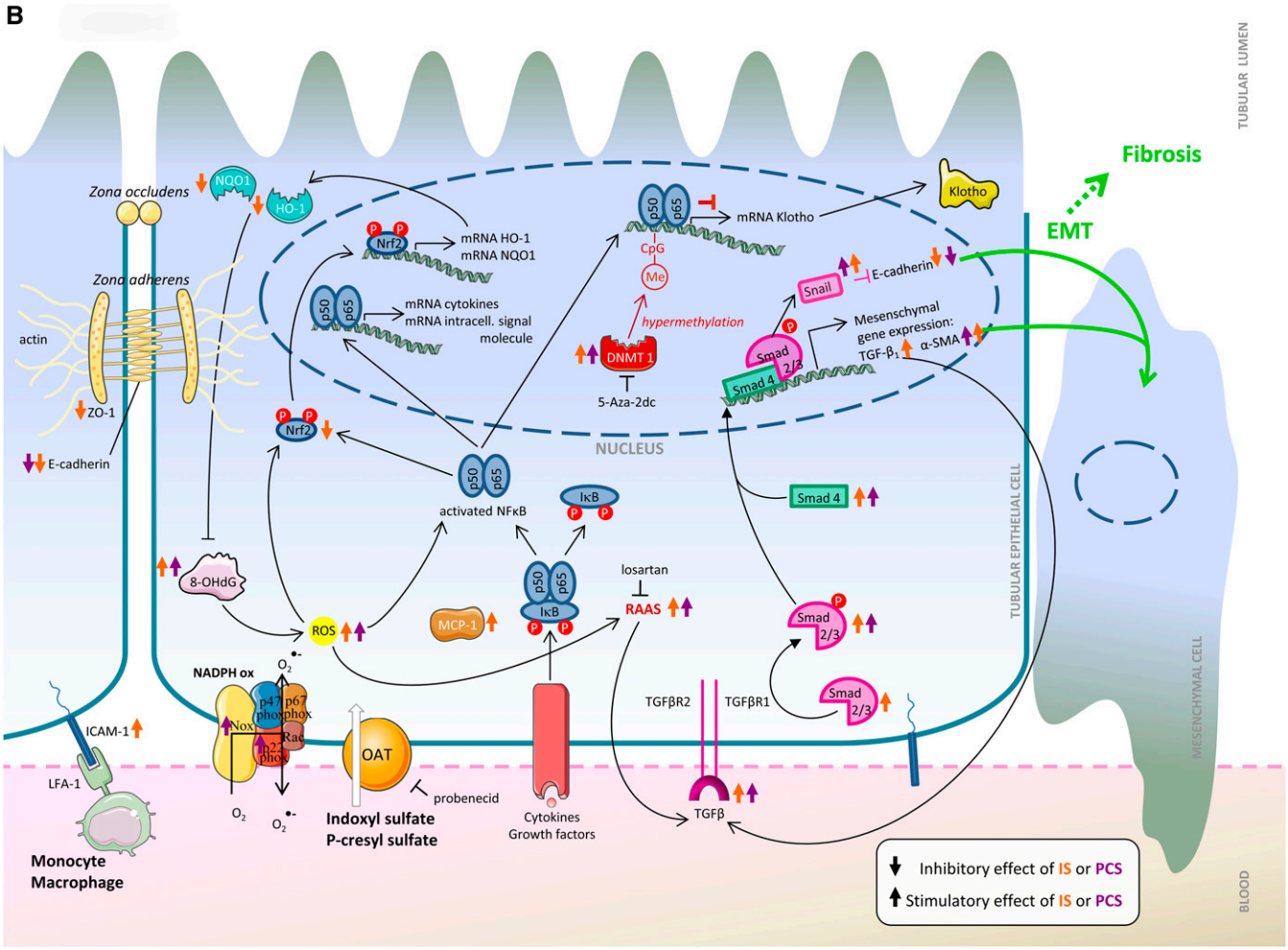

Figure 2. Continued.

difficult, were rejected as well as reviews, abstracts, and studies referring to conditions other than CKD, including AKI.

Only studies conforming to the preset target concentrations, defined as values not exceeding the highest levels obtained in uremia (reported in Table 1), were included.[4,20,77,78] Additional conditions for accepting a study were as follows: *in vitro* studies with use of medium containing concentrations of albumin corresponding to real-life *in vivo* conditions or animal studies with injection or oral administration of the molecules of interest or their precursors and the appropriate concentration of toxin and serum albumin. In the absence of albumin *in vitro*, the use of solute concentrations had to be in accordance with reported free toxin concentrations, which is also detailed in Table 2.

Based on this approach, two investigators independently selected the studies and extracted all data, with discrepancies resolved by a third investigator (Figure 1).

The studies were then checked for their content using a data extraction list conceived as reported in Supplemental Material 1, with specific attention to the experimental setting, the assessed problem, the molecule of interest, the applied concentrations, the presence (or absence) of appropriate concentrations of albumin, the involved pathways, and the successful application of neutralizing interventions.

From our analysis, it became clear that the quality of the retrieved publications was not always the same. However, to the best of our knowledge, there are no checklists available for evaluating quality or reliability of primary studies of biologic effects, the primary target of our search. Thus, we developed a quality score (Supplemental Material 2) that was given to each study based on the number of experiments (1 point if six or more experiments); confirmation of the obtained results by an alternative approach (same parameter, different methods, same pathway, different parameters; 1 point); proof of neutralization of the basic identified mechanism by the appropriate antibodies, counteractive agents, or other interventions (1 point); use of a real-life model (1 point; here, we excluded studies in specific genetic animal strains instead of wild type, especially because results between these two groups in our search were not always conformed[20,40,42,49]; also, specific cell types that are not necessarily representative of real-life conditions were excluded [*e.g.*, human umbilical vein endothelial cells], because the phenotype of venous endothelium

does not always match that of the arterial cell, which is where uremic vascular damage and atherosclerosis essentially take place[83–85]); and finally; performance of dose–response experiments but only if the other conditions were appropriate (in the presence of albumin or in the free concentration range; 1 point). Thus, a maximum quality score of 5 points could be obtained.

These quality scores were attributed independently by two of the authors and matched and double-checked by a third author in case of disagreement.

Because there are substantial ethical and practical obstacles to performing dose–response experiments for *in vivo* animal studies, 1 extra point was added to all publications containing animal studies as long as the same publication did not include *in vitro* dose–response studies as well.

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DISCLOSURES

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